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(54) **Methods and reagents for identifying bacteria.**

(57) Methods and reagents are provided for detecting polynucleotide sequences in bacteria using probes specific for gram-negative and gram-positive bacteria and other bacterial species or groups of species respectively. Also provided is a method for the amplification using primers specific for bacterial species.

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The present invention relates generally to methods and reagents for identifying and detecting gram-positive and gram-negative bacteria and other bacteria causing septicemia.

In order to successfully treat a disease caused by a bacterium the rapid and accurate detection and identification of the disease-causing bacterium is required. The detection and identification has traditionally been accomplished by pure culture isolation, followed by identification procedures that make use of knowledge of specimen source, growth requirements, visible (colony) growth features, microscopic morphology, staining reactions, and biochemical characteristics.

An important step in determining the identity of a bacterium is the Gram stain. This procedure involves treating a heat-fixed bacterial smear on a glass slide with the basic dye, crystal violet. All organisms take up the dye. The smear is then covered with Gram's iodine solution (3 percent iodine-potassium iodide in water or a weak buffer, pH 8.0, in order to neutralize acidity formed from iodine on standing). After a water rinse and decolorization with acetone, the preparation is washed thoroughly in water and counterstained with a red dye, usually safranin. The stained preparation is then rinsed with water, dried, and examined under oil using a light microscope.

Most bacteria can be differentiated into two groups by this stain. Gram-positive organisms stain blue, whereas about one-third of the cocci, one-half of the bacilli, and all spiral organisms stain red and are said to be gram-negative. This method, while effective, is very time consuming and involves many different procedures which present many opportunities for error. The Gram stain and other culture-based methods of detection require incubation of the sample with culture medium at least overnight in order to obtain a pure culture.

The presence of bacteria or fungi in the blood, commonly referred to as septicemia, can have severe and life-threatening clinical consequences. Septicemia can result in septic shock, which includes the following symptoms - hypotension, lactic acidosis, hypoxemia, oligouria, confusion, disseminated intravascular coagulation, gastrointestinal bleeding, disturbances of metabolism, and subtle skin lesions. As little as one colony-forming unit (CFU) may be present in a 30 ml blood sample in a patient with septicemia. Since culture is currently the most sensitive and commonly used method of detecting bacteria or fungi in the blood, treatment of suspected septicemia is often begun empirically, without waiting for the results of culture. It is clear that a rapid diagnostic method for detecting bacteria in the blood with the same sensitivity as culture would be a significant improvement over currently used methods.

Therefore, the present invention provides methods and reagents for the rapid detection and identification of bacteria causing septicemia. The detection is based upon the hybridization of nucleotide probes to nucleotide sequences as well as transcripts therefrom present in defined species or group of species but not in others.

In a preferred embodiment, a target region from genomic DNA or from a reverse transcript of 16S rRNA is amplified and the resultant amplified DNA is treated with a panel of probes which can hybridize to the DNA of a species or group of species of bacteria but not to others. The probes which sucessfully hybridize to the amplified DNA are determined and the bacterium is classified as either gram-positive or gram-negative or as a particular species or group of species depending on which probes hybridize to the amplified DNA.

Also defined and claimed herein are specific probes and their complements for identifying gram-negative and gram-positive and other bacteria causing septicemia.

The invention further contemplates the formulation and use of Polymerase Chain Reaction (PCR) kits containing universal bacterial primers for amplifying a specific universal target region of DNA for all bacteria and a panel of probes which hybridize to a nucleotide sequence which is unique to a species or group of species of bacteria within that target region.

Brief Description of the Figures

Fig. 1 shows two universal bacterial primers DG74 and RW01 which can be used to amplify a target region by PCR in gram-positive, gram-negative and other bacteria.

Fig. 2 shows a gram-positive specific probe RW03.

Fig. 3 shows four candidate gram-negative probes, RW04, DL04, DL05, and RDR278.

Fig. 4 shows two candidate universal bacterial probes RDR244 and RDR245.

Fig. 5 shows a Escherichia coli/enteric bacteria probe.

Fig. 6 shows a Bacteroides probe.

Table 1 summarizes the hybridization data on the Gram-negative probes RW04 and DL04.

Table 2 summarizes the results of testing probes RDR278 and DL04, the Gram-negative probes; RDR244 and RDR245, two candidate universal bacterial probes; and RDR279, the Bacteroides probe.

Table 3 shows a list of organisms tested with RDR244 and RDR245 as described in Example 6.
Table 4 shows a summary of data obtained with the E.coli/enteric bacteria probe RDR140KG.

Detailed Description of the Invention

The present invention relates to a method for determining the presence of and identification of bacteria by means of hybridizing probes to nucleotide sequences which are unique to either gram-positive or gram-negative bacteria or to a species or group of species of bacteria.

The use of specific polynucleotide sequences as probes for the recognition of infectious agents is becoming a valuable alternative to problematic immunological identification assays. For example, PCT publication W084/02721, published 19 July 1984 describes the use of nucleic acid probes complementary to targeted nucleic acid sequences composed of ribosomal RNA, transfer RNA, or other RNA in hybridization procedures to detect the target nucleic acid sequence. While the assay may provide greater sensitivity and specificity than known DNA hybridization assays, hybridization procedures which require the use of a complementary probe are generally dependent upon the cultivation of a test organism and are, therefore, unsuitable for rapid diagnosis. Probes can be used directly on clinical specimens if a means of amplifying the DNA target is available.

For use in the present invention, probes for bacterial species or groups of species causing septicemia include but are not limited to:
universal bacterial probes
Gram-negative probes
Gram-positive probes
Escherichia coli/enteric bacteria probes
Bacteroides probes

These probes would be useful in hybridizing to DNA or RNA amplified by the Polymerase Chain Reaction (PCR). PCR is a powerful technique that can be used for the detection of small numbers of pathogens whose in vitro cultivation is difficult or lengthy, or as a substitute for other methods which require the presence of living specimens for detection. In its simplest form, PCR is an in vitro method for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. A repetitive series of cycles involving template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase results in the exponential accumulation of a specific fragment whose termini are defined by the 5' ends of the primers. PCR reportedly is capable of producing a selective enrichment of a specific DNA sequence by a factor of 10^{12}. The PCR method is described in Saiki et al., (1985) Science 230, 1350-1354 and is the subject of U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159. This method has been used to detect the presence of the aberrant sequence in the beta-globin gene which is related to sickle cell anemia (Saiki et al., (1985) supra) and the human immunodeficiency virus (HIV) RNA (Byrne et al., (1988) Nuc. Acids Res. 16, 4165). However, before the method can be used, enough of the nucleotide sequence of the disease-associated polynucleotide must be known to design primers for the amplification, and to design probes specific enough to detect the amplified product.

The invention provides a method for determining the presence of a bacterial polynucleotide in samples suspected of containing said polynucleotide, wherein said polynucleotide contains a selected target region, said method comprising:

(a) amplifying the target region, if any, to a detectable level;

(b) incubating the amplified target region, if any, with a probe under conditions which allow specificity of hybrid duplexes; and

(c) detecting hybrids formed between the amplified target region, if any, and the probe.

In the above method, and as specific embodiments, the bacteria may be gram-positive or gram-negative or other defined bacterial species or group of species causing septicemia. Without being limited, the probe may be a universal bacterial probe, an E. coli/enteric probe, a gram-negative probe, a gram-positive probe or a Bacteroides probe or a combination of these probes.

the "target sequence". The nucleic acid contained in the sample may be first reverse transcribed into cDNA using Tth DNA polymerase as purified enzyme, if necessary, and then denatured, using any suitable denaturing method including physical, chemical, or enzymatic means, which are known to those of skill in

the art. A preferred physical means for strand separation involves heating the nucleic acid until it is completely (>99%) denatured. Typical heat denaturation involves temperatures ranging from about 80 °C to about 150 °C, for times ranging from about 5 seconds to 10 minutes using current technology.

The denatured DNA strands are then incubated with the selected oligonucleotide primers under hybridization conditions, conditions which enable the binding of the primers to the single oligonucleotide strands. As known in the art, the primers are selected so that their relative positions along a duplex sequence are such that an extension product synthesized from one primer, when it is separated from its complement serves as a template for the extension of the other primer to yield a replicate chain of defined length.

The primer must be sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact length of the primers will depend on many factors, including temperature, source of the primer and use of the method. For example, depending on the complexity of the target sequence, the oligonucleotide primer typically contains about 15-30 nucleotides, although it may contain more or fewer nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. The primers must be sufficiently complementary to selectively hybridize with their respective strands.

The primers used herein are selected to be "substantially" complementary to the different strands of each specific sequence to be amplified. The primers need not reflect the exact sequence of the template, but must be sufficiently complementary to selectively hybridize with their respective strands. Non-complementary bases or longer sequences can be interspersed into the primer, or the primer can contain a subset complementary to the specific sequence provided that the primer retains sufficient complementarity with the sequence of one of the strands to be amplified to hybridize therewith, and to thereby form a duplex structure which can be extended by the polymerizing means. The non-complementary nucleotide sequences of the primers may include restriction enzyme sites. Appending a restriction enzyme site to the end(s) of the target sequence is particularly helpful for subsequent cloning of the target sequence.

The oligonucleotide primers and probes for use in the present invention are shown in Figures 1-6. They may be prepared by any suitable method. Methods for preparing oligonucleotides of specific sequence are known in the art, and include, for example, cloning and restriction of appropriate sequences, and direct chemical synthesis. The primers may be labeled, if desired, by incorporating means detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means.

Template-dependent extension of the oligonucleotide primer(s) is then catalyzed by a polymerizing agent in the presence of adequate amounts of the four deoxyribonucleoside triphosphates (dATP, dGTP, dCTP, and dTTP) or analogs, in a reaction medium which is comprised of the appropriate salts, metal cations, and pH buffering system. Suitable polymerizing agents are enzymes known to catalyze primer- and template-dependent DNA synthesis. Known DNA polymerases include, for example, E. coli DNA polymerase I or its Klenow fragment, T_4 DNA polymerase, Taq DNA polymerase, Tth DNA polymerase from Thermus thermophilus and DNA polymerase from Thermococcus litoralis. The reaction conditions for catalyzing DNA synthesis with these DNA polymerases are well known in the art.

The products of the synthesis are duplex molecules consisting of the template strands and the primer extension strands, which include the target sequence. These products, in turn, serve as templates for another round of replication. In the second round of replication, the primer extension strand of the first cycle is annealed with its complementary primer; synthesis yields a "short" product which is bounded on both the 5'-and the 3'-ends by primer sequences or their complements. Repeated cycles of denaturation, primer annealing, and extension result in the exponential accumulation of the target region defined by the primers. Sufficient cycles are run to achieve the desired amount of polynucleotide containing the target region of nucleic acid. The desired amount may vary, and is determined by the function which the product polynucleotide is to serve.

The PCR method can be performed in a number of temporal sequences. For example, it can be performed step-wise, where after each step new reagents are added, or in a fashion where all of the reagents are added simultaneously, or in a partial step-wise fashion, where fresh reagents are added after a given number of steps.

In a preferred method, the PCR reaction is carried out as an automated process which utilizes a thermostable enzyme. In this process the reaction mixture is cycled through a denaturing step, a primer annealing step, and a synthesis step. A DNA thermal cycler specifically adapted for use with a thermostable enzyme may be employed, which utilizes temperature cycling without a liquid-handling system, thereby eliminating the need to add the enzyme at every cycle. This type of machine is commercially available.

After amplification by PCR, the target polynucleotides may be detected directly by gel analysis provided the target DNA is efficiently amplified and the primers are highly specific to the target region to be

amplified. To assure PCR efficiency, glycerol and other related solvents such as dimethyl sulfoxide, can be used to increase the sensitivity of the PCR at the amplification level and to overcome problems pertaining to the sequencing of regions of DNA having a strong secondary structure. These problems may include (1) low efficiency of the PCR, due to a high frequency of templates that are not fully extended by the polymerizing agent or (2) incomplete denaturation of the duplex DNA at high temperature, due to high GC content. The use of such solvents can increase the sensitivity of the assay at the level of amplification to approximately several femtograms of DNA (which is believed to correspond to a single bacterial cell). This level of sensitivity eliminates the need to detect amplified target DNA using a probe, and thereby dispenses with the requirements for radioactive probes, gel electrophoresis, Southern blotting, filter hybridization, washing and autoradiography. The concentration range for glycerol is about 5%-20% (v/v), and the DMSO concentration range is about 3% - 10% (v/v).

Alternatively, and in accordance with the present invention the target polynucleotides may be detected by hybridization with a probe polynucleotide which forms a stable hybrid with that of the target sequence under stringent to low stringency hybridization and wash conditions. If it is expected that the probes will be completely complementary (i.e., about 99% or greater) to the target sequence, stringent conditions will be used. If some mismatching is expected, for example if variant strains are expected with the result that the probe will not be completely complementary, the stringency of hybridization may be lessened. However, conditions are chosen which rule out nonspecific/adventitious binding. Conditions which affect hybridization and which select against nonspecific binding are known in the art. Generally, lower salt concentration and higher temperature increase the stringency of binding. For example, it is usually considered that stringent conditions are incubation in solutions which contain approximately 0.1 x SSC, 0.1% SDS, at about 65 °C incubation/wash temperature, and moderately stringent conditions are incubation in solutions which contain approximately 1-2 X SSC, 0.1% SDS and about 50 °-65 °C incubation/wash temperature. Low stringency conditions are 2 X SSC and about 30 °-50 °C.

An alternate method of hybridization and washing is to perform a low stringency hybridization (5x SSPE, 0.5% SDS) followed by a high stringency wash in the presence of 3M tetramethylammonium chloride (TMACl). The effect of the TMACl is to equalize the relative binding of A-T and G-C base pairs so that the efficiency of hybridization at a given temperature is a function of the length of the polynucleotide. Using TMACl, it is possible to vary the temperature of the wash to achieve the level of stringency desired. (See Base composition-independent hybridization in tetramethylammonium chloride: A method for oligonucleotide screening of highly complex gene libraries; Wood, et al. (1985) Proc. Natl. Acad. Sci. USA 82, 1585-1588).

Probes for bacterial target sequences may be derived from the 16S rRNA gene sequences or their complements. The probes may be of any suitable length which span the target region, but which exclude the primers, and which allow specific hybridization to the target region. Generally, the probes will have at least 14 nucleotides, preferably at least 18 nucleotides, and more preferably at least 20 to 30 nucleotides of either of the complementary DNA strands. In fact, the target sequence can come from either complementary DNA strands. If there is to be complete complementarity, i.e., if the strain contains a sequence identical to that of the probe, since the duplex will be relatively stable under even stringent conditions, the probes may be short, i.e., in the range of about 10-30 basepairs. If some degree of mismatch is expected with the probe, i.e., if it is suspected that the probe will hybridize to a variant region, the probe may be of greater length, since length seems to counterbalance some of the effect of the mismatch(es). The probe may be formed from a subset of the target region and therefore need not span the entire target region. Any subset of the target region can be used in constructing the probe provided the probe by hybridizing to that portion of the target region will specifically identify the target region. If desired, the probe may also be labeled. A variety of labels which would be appropriate, as well as methods for their inclusion in the probe are known in the art, and include, for example, radioactive atoms, such as ^{32}P, or other recognizable functionalities, e.g., biotin (preferably using a spacer arm), fluorescent dyes, electron-dense reagents, enzymes capable of forming easily detectable reaction products (e.g., alkaline phosphatase, and horseradish peroxidase), or antigens for which specific antisera or monoclonal antibodies are available.

Analysis of the nucleotide sequence of the target region may be by direct analysis of the PCR amplified

preferable to subject the products to size analysis as well as hybridization with the probe. Methods for determining the size of nucleic acids are known in the art, and include, for example, gel electrophoresis, sedimentation in gradients, and gel exclusion chromatography

The presence of the target sequence in a biological sample is detected by determining whether a hybrid has been formed between the probe and the nucleic acid subjected to the PCR amplification techniques. Methods to detect hybrids formed between a probe and a nucleic acid sequence are well-known in the art. For example, an unlabeled sample may be transferred to a solid matrix to which it binds, and the bound sample subjected to conditions which allow specific hybridization with a labeled probe; the solid matrix is then examined for the presence of the labeled probe. Alternatively, if the sample is labeled, an unlabeled probe is bound to the matrix, and after exposure to the appropriate hybridization conditions, the matrix is examined for the presence of a label. Saiki et al., (1988) Proc. Natl. Acad. Sci. USA 86, 6230-6234 describe methods of immobilizing multiple probes on a solid support and using hybridization to detect the amplified target polynucleotides of interest. The latter procedure is well suited to the use of a panel of probes which can provide different levels of identification of an amplified target DNA, depending on the type of information desired. In another alternative procedure, a solution phase sandwich assay may be used with labeled polynucleotide probes, and the methods for the preparation of such probes are described in U.S. Patent No. 4,820,630, issued April 11, 1989.

Also within the scope of the present invention are PCR kits for use in carrying out any of the aforementioned PCR processes. The PCR kits for the detection of bacteria comprise a first container and a second container wherein the first container contains primers capable of amplifying a target region of a polynucleotide sequence within bacteria, and the second container contains one or more probes capable of hybridizing to the amplified target nucleic acid sequence. Preferably, the primers used are these as shown in Figure 1 and the probe is selected from the probes shown in Figures 2-6. Either of these may or may not be labeled. If unlabeled, the ingredients for labeling may also be included in the kit. The kit may also contain other suitably packaged reagents and material needed for the particular hybridization protocol, for example, standards, and/or polymerizing agents, as well as instruction for conducting the test.

In use, the components of the PCR kit, when applied to a nucleic acid sample, create a reagent mixture which enables the detection and amplification of the target nucleic acid sequence. The reagent mixture thus includes the components of the kit as well as a nucleic acid sample which contains the polynucleotide chain of interest.

By bay of further specificity, the following probe and primer nucleotide base pair data is provided:

Probe RDR245, Fig. 4, corresponds to the complement of nucleotide base numbers 1369-1395 in the E. coli. 16S ribosomal RNA gene as specified in the reference of Neefs et al. (infra)

Primer RW01, Fig. 1, corresponds to nucleotide base numbers 1170-1189 in the E. coli 16S ribosomal RNA gene as specified in Neefs reference.

Primer D674, Fig. 1, corresponding to the complement of nucleotide base numbers 1522-1540 in the E. coli 16S ribosomal RNA gene as specified in Neefs reference.

Probe RW03, Fig. 2, corresponding to nucleotide base number 1190-1217 in the E. coli 16S ribosomal RNA gene as specified in Neefs reference.

Probes DL04 and RDR278, Fig. 3, corresponding to the complement of nucleotide base number 1190-1217 in the E. coli 16S ribosomal RNA gene as specified in Neefs reference.

Probe RDR140 KG, Fig. 5, corresponding to nucleotide base number 1458-1482 in the E. coli 16S ribosomal RNA gene as specified in Neefs reference.

Probe RDR279, Fig. 6, corresponding to the complement of nucleotide base number 1190-1217 in the E. coli 16S ribosomal RNA gene as specified in Neefs reference.

Oligonucleotide probes based on the 16S rRNA gene for the detection of nucleic acids from various microorganisms have been described in the scientific literature. For example, universal bacterial probes have been described by Wilson, et al ("Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction", Kenneth Wilson, Rhonda Blitchington, and Ronald Greene (1990), Journal of Clinical Microbiology, 28, 1942-1946) and Chen, et al ("Broad range DNA probes for detecting and amplifying eubacterial nucleic acids", Kui Chen, Harold Neimark, Peter Rumore, and Charles Steinman, (1989) FEMS microbiology letters, 57, 19-24). Examples of genus- and species-specific probes have been described by Barry, et al ("A general method to generate DNA probes for microorganisms", Tom Barry, Richard Powell, Frank Gannon (1990), Biotechnology 8, 233-236), Atlas and Bej ("Detecting bacterial pathogens in environmental water samples by using PCR and gene probes", Ronald Atlas and Asim Bej, in "PCR protocols: A guide to methods and applications," (1990) pp. 399-406 (Jnis M.A., ed.), Academic Press, Inc.), and in Genprobe international patent application with Publ. No. W088/03957. The invention claimed in this application differs from these inventions in the range of bacteria detected. The gram-positive and gram-negative probes detect a range of different bacterial genera and are therefore more specific than universal bacterial probes and more broad than genus- or species-specific probes. Using a panel including a universal bacterial probe, gram-positive and gram-negative probes and species or group specific probes, it is possible to obtain a

more reliable detection of a bacterium than the use of a collection of genus- or species-specific probes, since it is possible for the universal bacterial and gram-negative or gram-positive probes to detect bacteria which are not detected by any of the more specific probes. The panel described also provides more clinically useful information than a single universal bacterial probe; since different antibiotic therapy is recommended for gram-negative versus gram-positive bacterial infections.

The following examples are intended to be illustrative of the various methods and compounds of the invention.

Example 1

Methods used to design probes of subsequent examples.

The candidate Gram-negative probes RW04, DL04, DL05, and RDR278, the Gram-positive probe RW03, and the Bacteroides probe RDR279 were designed from data in the Genbank or EMBL nucleotide sequence libraries (Dams, et al., "Compilation of small ribosomal subunit RNA sequences", (1988) Nucleic Acids Research, Vol. 16, Supplement and Neefs, et al, "Compilation of small ribosomal subunit RNA sequences", (1990) 18, 2237-2317, Supplement and in a paper by C. Woese (C. R. Woese, "Bacterial evolution", (1987) Microbiological Reviews, 51 (2), 221-271).

The location of the probes was chosen based on the finding of regions in the 16S rRNA gene which contain "sequence signatures" unique to the various groups of bacteria (Woese reference). All six of these probes are located in the same region of the gene. The nucleotide sequence of the probes was designed based on the sequences available for each group of bacteria to be detected and compared to corresponding sequences in groups of bacteria that were to be excluded. For example, the Gram-positive probe was designed to match most of the sequences found in most Gram-positive bacteria and to differ from the corresponding sequences in Gram-negative bacteria.

The candidate universal bacterial probes RDR244 and RDR245 correspond to a highly conserved region of the 16S rRNA gene. Most of the probe sequence in this region is present in most of the bacterial species for which sequence information is available and is not present in the nuclear or mitochondrial DNA of eukaryotic species.

In addition, each of the oligonucleotides described above was examined for self complementarity (ability to form base pairs with itself) using a computer program called FOLD in the University of Wisconsin series of programs. The position of the oligonucleotide probe was chosen to minimize the formation of secondary structure where it was possible to do so while still maintaining the desired specificity.

The E. coli/enteric bacteria probe was designed from data in Genbank. The probe was designed using the following steps:

First, the nucleotide sequence from bp 1430 to 1536 (as specified in the Neefs reference) (within the 370 bp region bounded by amplification primers RW01 and DG74) for E. coli and Proteus vulgaris was compared to that of a panel of nonenteric species, including Neisseria gonorrhoeae, Pseudomonas aeruginosa, and Pseudomonas testosteroni. Regions where differences in the sequence occurred were noted and used to design a candidate probe.

Second, the candidate probe was compared with the corresponding nucleotide sequence of more phylogenetically diverse species listed in Genbank or EMBL to ensure that the candidate oligonucleotide would not detect other species.

Third, the oligonucleotide was examined for self complementarity (ability to form base pairs with itself) using a computer program called OLIGO, (National Biosciences, Hamel, NN). The position of the oligonucleotide probe was chosen to minimize the formation of secondary structure where it was possible to do so while still maintaining the desired specificity.

Example 2

Detection of Gram-negative bacteria Using PCR and Probe DL04

gram-positive and gram-negative bacteria.

10x standard PCR buffer	10.0 ml
50 mM MgCl	1.0 ml

dNTP's (2.5 mM total dNTP's)	2.5 ml
primer RW01 (50 mM)	1.0 ml
primer DG74 (50 mM)	1.0 ml
H_2O	35.0 ml
Taq DNA polymerase (5 U/ml)	0.5 ml

The 10x standard PCR buffer contains:
100 mM Tris-HCl, pH8.3
500 mM KCl
15 mM MgCl

A. 50 ml of a gram-negative bacterial DNA sample was mixed together with 50 ml of the PCR 2x mix.

The reaction mixture was placed in a 0.5 ml microfuge tube and the tube was placed in a thermal cycler manufactured by Perkin-Elmer. A two-step PCR cycle was used and the thermocycler was set as follows:

1. Time delay file - 5 minutes at 95 ° C
2. Thermocycle file - 95 ° C for 25 seconds 55 ° C for 25 seconds, each incubation for 25 to 35 cycles.
3. Time delay file - 10 minutes at 72 ° C.

B. Detection of amplified products

After the amplification reaction is complete, 5 ml of the 100 ml PCR reaction was mixed with 1 ml of 10x DNA dye buffer (50% sucrose, 10mM Tris, pH 7.5, 1 mM EDTA, 1.0% SDS, 0.05% bromphenol blue). The sample was loaded onto a 2% Nusieve agarose, 0.5% Seakem agarose, 1x TBE (45 mM Tris-borate, 1mM EDTA) gel. After running the bromphenol blue dye front to the bottom of the gel, the gel was stained with ethidium bromide (5 mg/ml), washed in water and photographed under UV light using a Polaroid camera and an orange filter.

The size of the PCR product is approximately 370 bp.

C. Transfer of amplified DNA to nylon membrane

After photography of the gel, the gel was soaked in 0.25 N HCl for 10 minutes at room temperature. The gel was rinsed in water and then soaked in solution of 0.5 N NaOH, 1.5 M NaCl for 30 minutes. The gel was then rinsed in water and then soaked in a solution of 1 M Tris, pH 7.5, 1.5 M NaCl for 30 minutes.

DNA was then transferred to a nylon membrane (Pall Biodyne) presoaked in water by one of two ways: (1) vacuum transfer using a Stratagene Stratavac vacuum blotter or (2) capillary transfer by the method of Southern.

After transfer, DNA was fixed to the membrane using UV light in a Stratagene Stratalinker.

D. Radioactive labeling of oligonucleotide probe DL04 (Figure 3).

Oligonucleotide DL04 was labeled using T_4 polynucleotide kinase in the following reaction mix:

γ-32-P ATP	10 ml
10x kinase buffer	2.5 ml
oligonucleotide (10mM)	2.0 ml
H_2O	8.5 ml
T4 polynucleotide kinase	2.0 ml

10x kinase buffer contains:
500 mM Tris, pH 8
100 mM MgCl
50 mM DTT

The kinase reaction mixture was incubated for 30 minutes at 37 ° C. 5.6 of 0.25 M EDTA and 169.4 ml of H_2O were added to stop the reaction. This mixture was loaded onto a 1.0 ml capacity column of Biogel P4 and spun in a tabletop centrifuge for 5 minutes at 5,000 rpm to separate the labeled oligonucleotide from the unincorporated radioactivity. 1ml of the eluate from the column was counted in a scintillation counter without added scintillation fluid (Cerenkov counting) to obtain an estimate of the level of incorporation of radioactivity. A volume giving approximately 1 x 10^6 cpm was used for each blot in the subsequent hybridization.

E. Hybridization of probes with DNA

The DNA blots were prehybridized in a mixture of 5x SSPE, 0.5% SDS at 60 ° C (1X SSPE = 0.18 M NaCl, 10 mM $NaPO_4$, pH 7.4, 1 mM EDTA). The labeled oligonucleotide probe was added to 7.5 ml of 5x SSPE, 0.5% SDS and mixed. The solution was added to the plastic bag containing the presoaked blot. The blot was incubated for 1 to 18 hours at 60 ° C.

The blot was removed from the plastic bag and placed in a solution of 2x SSPE, 0.1% SDS and washed for 10 minutes at room temperature. The blot was then washed in a solution of 3 M tetramethylammonium chloride (TMACl), 50 mM Tris, pH8 and 0.2% SDS for 10 minutes at 64 ° C for gram-negative probe DL04.

The blot was air-dried and wrapped in Saran wrap and placed in a X-ray film holder with a sheet of Kodak XAR-5 X-ray film with or without an intensifying screen for 1 to 72 hours at -70 ° C.

Example 3

Detection of Gram-positive bacteria using PCR and Probe RW03

Gram-positive bacteria were detected using the same methods and materials as Example 2 except as follows:

50 ml of a gram-positive containing DNA sample was added to the PCR 2x mix.

The probe used was the gram-positive specific probe RW03 (Figure 2). In step E, when the blot was washed in a solution of 3M TMACl, 50 mM Tris, pH8 and 0.2% SDS, it was done at 62 ° C instead of 64 ° C as was done for the gram-negative test.

Example 4

Detection of Gram-negative bacteria - comparison of probes RW04 and DL04

Candidate Gram-negative probe RW04 was labeled with ^{32}P and hybridized to PCR products from various bacterial DNA's as described for Gram-negative probe DL04 in Example 2, except that the wash in TMACl was done at 62 ° C. The Southern blot results of the hybridizations are summarized in Table 1. The data show that the hybridization results obtained by the two probes are different even though both probes were designed to be Gram-negative "universal" probes. RW04 gave a positive signal for many Gram-positive species it should not have detected; while DL04 gave positive signals for only the Gram-negative species it should have detected (with the exception of T. maritima and T. thermophilus, which are not human pathogens). DL04 was therefore selected as a probe useful for detecting Gram-negative bacteria. Further testing (Table 2) indicated that DL04 did not detect all gram-negative species. A second candidate Gram-negative probe, RDR278, was tested as follows in Example 5.

Example 5

Detection of Gram-negative bacteria with probe RDR278

Gram-negative bacteria were detected using the same methods and materials as Example 2 including the wash in TMACl which was done at 64 ° C.

Gram-negative probe RDR278 was labeled with ^{32}P and hybridized to PCR products from various bacterial DNA's. The data are presented in Table 2. RDR278 gave a positive hybridization signal for most of the species not detected by DL04. The exception among the species tested was Bacteroides fragilis, for which a separate probe was designed. Therefore, it is observed that the combination of Gram-negative probes DL04 and RDR278 detect the majority of Gram-negative bacteria tested.

Example 6

64 ° C.

Candidate Universal bacterial probes RDR244 and RDR245, corresponding to a highly conserved region in the 16S rRNA gene, were labeled with ^{32}P and hybridized to PCR products from various bacterial DNA's

Table 3 summarizes the bacterial DNA's tested by Southern blot hybridization with RDR244 and RDR245. The probes performed differently even though both probes were designed to detect any bacterial species. It is observed that, among the bacterial species tested, RDR244 detected all but two species: Peptostreptococcus magnus (No. 29) and P. anaerobius (No. 28). RDR245 detected all of the bacterial species tested. Therefore, RDR245 was selected as the universal bacterial probe.

Example 7

Detection of Bacteroides fragilis with probe RDR279

The methods and materials of Example 2 were used including the wash in TMACl, which was done at 64 ° C.

Probe RDR279, corresponding to a region which is a sequence signature for Bacteroides (ref.), was labeled with ^{32}P and hybridized to PCR products from various bacterial DNA's. Table 2 summarizes the results of testing of RDR279 against other bacterial species. The probe detected Bacteroides fragilis and did not give a reaction with any of the other bacterial species tested.

Example 8

Detection of Escherichia coli/enteric bacteria with probe RDR140KG

E. coli/enteric bacteria were detected using the same methods and materials as in Example 2 including the wash in TMACl which was done at 66 ° C. The results for the E. coli/enteric bacteria probe are shown in Table 4 which confirm that with this probe only the intended species were detected.

The probes described above are applied to the detection of septicemia by using them in combination to detect and identify what bacterium is present in a blood sample. All of the probes described above, as well as additional probes, can be arranged in a reverse dot blot format, as described by Saiki, et al. The probes are immobilized on a solid support such as nylon membrane or microtiter plate. The amplified DNA is hybridized to each of the probes at the same time in an aqueous solution. The pattern of the signals from each of the probes indicates the identity of the target DNA. For example, if the DNA is from a Gram-negative bacterium, the amplified DNA will only react with the universal bacterial probe and one of the Gram-negative probes. If the DNA is from a Gram-positive species, it will give a positive reaction only with the universal bacterial probe and the Gram-positive probe. If the DNA is from a Bacteroides species, it will give a positive signal with the universal bacterial probe and the Bacteroides probe. If the DNA is from a bacterium which is neither Gram-negative nor Gram-positive (such as T. pallidum, a spirochete) it will react only with the universal bacterial probe. If there is no bacterial DNA present, none of the probes will give a positive signal.

Although the foregoing invention has been described in some detail for the purpose of illustration, it will be obvious that changes and modifications may be practiced within the scope of the appended claims by those of ordinary skill in the art.

Table 1

RW04 data

HYBRIDIZATION RESULTS			
	Gram+/Gram-	Probes	
STRAIN		DL04	RW04
Thermotoga maritima	Neither	-	N.D.
Thermus thermophilus	Neither	-	N.D.
Shigella boydii	-	+	N.D.
S. dysenteriae	-	+	N.D.
S. flexneri	-	+	N.D.
S. sonnei	-	+	N.D.
Enterobacter aerogenes	-	+	+
Klebsiella pneumoniae	-	+	+
Salmonella typhimurium	-	+	+
Serratia marcescens	-	+	+
Pseudomonas aeruginosa	-	+	+
Treponema vincentii	Neither	+	+
Thermus aquaticus	Neither	+	-
Acetobacter sp.	-	+	+
Acetobacter sp.	-	+	+
Staphylococcus aureus	+	-	+
Clostridium perfringens	+	-	+
Micrococcus lysodeikticus	+	-	-
Bacillus subtilis	+	-	+
B. amyloliquefaciens	+	-	+
Streptomyces hygroscopicus	+	+	+
N.D. indicates not done			

Table 2a

STRAIN	REFERENCE	Gram-	Gram-	Bacteroides	universal	universal
		DL04	RDR278	RDR279	RDR244	RDR245
GRAM-POSITIVE						
Actinomyces israelii	ATCC 12102	±	-	-	+	+
Aerococcus viridans	ATCC 11563	-	-	-	+	+
Bacillus amyloliquefaciens	H	-	N.D.	N.D.	N.D.	N.D.
Bacillus subtilis	ATCC 6051	-	-	-	+	+
B. subtilis		-	N.D.	N.D.	N.D.	N.D.
Bifidobacterium adolescentis	ATCC 15703	-	-	-	+	+
Brevibacterium linens	ATCC 9172	-	-	-	+	+
Clostridium innocuum	ATCC 14501	-	-	-	+	+
C. perfringens	ATCC 13124	-	-	-	+	+
C. perfringens	Sigma	-	N.D.	N.D.	N.D.	N.D.
Corynebacterium genitalium	ATCC 33030	±	-	-	+	+
C. pseudotuberculosis	ATCC 19410	-	-	-	+	+
C. xerosis	ATCC 373	±	-	-	+	+
Deinococcus radiopugnans	ATCC 19172	-	-	-	+	+
Enterococcus avium	ATCC 14025	-	-	-	+	+
E. faecalis	ATCC 19433	-	-	-	+	+
E. faecium	ATCC 19434	-	-	-	+	+
Erysipelothrix rhusiopathiae	ATCC 19414	-	-	-	+	+
Gardnerella vaginalis	ATCC 14018	-	-	-	+	+
Gemella haemolysans	ATCC 10379	±	-	-	+	+
Lactobacillus acidophilus	ATCC 4356	-	-	-	+	+
L. brevis	ATCC 14869	-	-	-	+	+
L. jensenii	ATCC 25258	-	-	-	+	+
Lactococcus lactis cremoris	ATCC 19257	-	-	-	+	+
L. lactis lactis	ATCC 19435	-	-	-	+	+
Leuconostoc paramesenteroides	ATCC 33313	-	-	-	+	+
Listeria monocytogenes	ATCC 15313	-	-	-	+	+
Micrococcus luteus	ATCC 4698	N.D.	-	-	+	+
M. lysodeikticus	Sigma	-	N.D.	N.D.	N.D.	N.D.
Mycobacterium bovis		-	N.D.	N.D.	N.D.	N.D.
M. gordonae	ATCC 14470	+	-	-	+	+

Table 2b

STRAIN	REFERENCE	Gram-	Gram-	Bacteroides	universal	universal
		DL04	RDR278	RDR279	RDR244	RDR245
GRAM-POSITIVE						
M. smegmatis	ATCC 19420	+	-	-	+	+
M. tuberculosis		-	N.D.	N.D.	N.D.	N.D.
Mycoplasma genitalium	ATCC 33530	+	-	-	+	+
M. hominis	ATCC 23114	-	-	-	+	+
M. pneumoniae	ATCC 15531	-	-	-	+	+
Pediococcus acidilactici	ATCC 33314	-	-	-	+	+
Peptostreptococcus anaerobius	ATCC 27337	-	-	-	-	+
P. magnus	ATCC 15794	±	-	-	-	+
Propionibacterium acnes	ATCC 6919	-	-	-	+	+
Staphylococcus aureus	ATCC 12598	-	-	-	+	+
S. aureus	ATCC 33589	-	N.D.	N.D.	N.D.	N.D.
S. aureus	ATCC 25923	-	N.D.	N.D.	N.D.	N.D.
S. epidermidis	ATCC 14990	-	-	-	+	+
Streptococcus agalactiae	ATCC 13813	-	-	-	+	+
S. bovis	ATCC 33317	-	-	-	+	+
S. dysgalactiae	ATCC 43078	-	-	-	+	+
S. equinus	ATCC 9812	-	-	-	+	+
S. intermedius	ATCC 27335	-	-	-	+	+
S. mitis	ATCC 33399	-	-	-	+	+
S. mutans	ATCC 25175	-	-	-	+	+
S. pneumoniae	ATCC 33400	-	-	-	+	+
S. pyogenes	ATCC 12344	-	-	-	+	+
S. pyogenes	ATCC 12344	-	N.D.	N.D	N.D.	N.D.
S. salivarius	ATCC 13419	-	-	-	+	+
S. sanguis	ATCC 10556	-	-	-	+	+
S. uberis	ATCC 19436	-	-	-	+	+
Streptomyces griseinus	ATCC 23915	±	-	-	+	+
S. hygroscopicus	21705	±	N.D.	N.D.	N.D.	N.D.
Ureaplasma urealyticum	ATCC 27618	+	-	-	+	+

Table 2c

ORGANISM	REFERENCE	Gram-	Gram-	Bacteroides	Universal	Universal
		DL04	RDR278	RDR279	RDR244	RDR245
GRAM-NEGATIVE						
Acinetobacter calcoaceticus	ATCC 23055	+	±	-	+	+
Acinetobacter lwoffi	ATCC 15309	+	±	-	+	+
Achromobacter xerosis	ATCC 14780	+	±	-	+	+
Aeromonas hydrophila	ATCC 7966	+	±	-	+	+
Agrobacterium radiobacter	ATCC 19358	±	+	-	+	+
Alcaligenes denitrificans	ATCC 27061	-	+	-	+	+
A. faecalis	ATCC 8750	-	+	-	+	+
Bacteroides fragilis	ATCC 25285	-	-	+	+	+
Branhamella catarrhalis	ATCC 25238	+	±	-	+	+
Campylobacter fetus	ATCC 27374	+	+	-	+	+
C. jejuni	ATCC 33560	+	+	-	+	+
Chromobacterium violaceum	ATCC 12472	-	+	-	+	+
Citrobacter freundii	ATCC 8090	+	±	-	+	+
Derxia gummosa	ATCC 15994	-	+	-	+	+
Edwardsiella tarda	ATCC15947	+	±	-	+	+
Eikenella corrodens	ATCC 23834	-	+	-	+	+
Enterobacter aerogenes	ATCC 13048	+	N.D.	N.D.	N.D.	N.D.
Enterobacter cloacae	ATCC 13047	+	±	-	+	+
Escherichia coli	ATCC 11775	+	±	-	+	+
Flavobacterium meningosepticum	ATCC 13253	N.D.	N.D.	N.D.	N.D.	N.D.
Haemophilus ducreyi	ATCC 33940	+	±	-	+	+
Haemophilus influenzae	ATCC 33391	-	+	-	+	+
H. influenzae	ATCC 33391	+	N.D.	N.D.	N.D.	N.D.
Kingella kingae	ATCC 23330	+	±	-	+	+
Klebsiella pneumoniae	ATCC 13883	-	-	-	+	+
K. pneumoniae	CMCC 151	+	N.D.	N.D.	N.D.	N.D.
Legionella bozemanii	ATCC 33217	+	±	-	+	+
Legionella pneumophila	ATCC 33152	+	±	-	+	+
Moraxella osloensis	ATCC 19976	+	±	-	+	+
Morganella morganii	ATCC 25830	-	N.D.	N.D.	N.D.	N.D.
Neisseria gonorrhoeae	ATCC 19424	-	+	-	+	+

Table 2d

STRAIN	REFERENCE	Gram- DL04	Gram- RDR278	Bacteroides RDR279	universal RDR244	universal RDR245
GRAM-NEGATIVE						
N. gonorrhoeae	CMCC 2783	N.D.	N.D.	N.D.	N.D.	N.D.
N. meningitidis	ATCC 13077	-	+	-	+	+
N. meningitidis	CMCC 2801	N.D.	N.D.	N.D.	N.D.	N.D.
Paracoccus denitrificans	ATCC 17741	+	+	-	+	+
Proteus mirabilis	ATCC 29906	+	±	-	+	+
Providencia stuartii	ATCC 29914	+	±	-	+	+
Pseudomonas aeruginosa	ATCC 10145	+	±	-	+	+
P. aeruginosa	ATCC 27853	+	N.D.	N.D.	N.D.	N.D.
Pseudomonas putida	ATCC 12633	+	±	-	+	+
Rahnella aquatilis	ATCC 33071	+	±	-	+	+
Rhodospirillum rubrum	ATCC 11170	-	±	-	+	+
Salmonella typhimurium	CMCC 2	+	N.D.	N.D.	N.D.	N.D.
Serratia marcescens	ATCC 13880	+	±	-	+	+
S. marcescens	CMCC 186	+	N.D.	N.D.	N.D.	N.D.
Shigella boydii		+	N.D.	N.D.	N.D.	N.D.
S. dysenteriae		+	N.D.	N.D.	N.D.	N.D.
S. flexneri		+	N.D.	N.D.	N.D.	N.D.
S. sonnei		+	N.D.	N.D.	N.D.	N.D.
Vibrio parahaemolyticus	ATCC 17802	N.D.	N.D.	N.D.	N.D.	N.D.
Yersinia enterocolitica	ATCC 9610	+	±	-	+	+

Table 3

	Organism
1	*Actinomyces israelii*
2	*Aerococcus viridans*
3	*Bacillus subtilis*
4	*Bifidobacterium adolescentis*
5	***Brevibacterium linens***
6	*Clostridium innocuum*
7	*Clostridium perfringens*
8	*Corynebacterium genitalium*
9	*Corynebacterium pseudotuberculosis*
10	*Corynebacterium xerosis*
11	*Deinococcus radiopugnans*
12	*Enterococcus avium*
13	*Enterococcus faecalis*
14	*Enterococcus faecium*
15	*Erysipelothrix rhusiopathiae*
16	*Gemella haemolysans*
17	*Lactobacillus acidophilus*
18	*Lactobacillus brevis*
19	*Lactobacillus jensenii*
20	*Lactococcus lactis cremoris*
21	*Lactococcus lactis lactis*
22	*Leuconostoc paramesenteroides*
23	*Listeria monocytogenes*
24	*Mycobacterium gordonae*
25	*Mycobacterium smegmatis*
26	*Paracoccus denitrificans*
27	*Pediococcus acidilactici*
28	*Peptostreptococcus anaerobius*
29	*Peptostreptococcus magnus*
30	*Propionibacterium acnes*
31/32	*Staphylococcus aureus (2)*
33	*Staphylococcus epidermidis*
34	*Streptococcus agalactiae*
35	*Streptococcus bovis*
36	*Streptococcus dysgalactiae*
37	*Streptococcus equinus*
38	*Streptococcus intermedius*
39	*Streptococcus mitis*
40	*Streptococcus mutans*
41	*Streptococcus pneumoniae*
42	*Streptococcus pyogenes*
43	*Streptococcus salivarius*
44	*Streptococcus sanguis*
45	*Streptococcus uberis*

Table 4

STRAIN	REFERENCE	PROBE
		RDR140
		E. coli
Neisseria gonorrhoeae	CMCC 2783	-
	ATCC 19424	-
	31917	-
	31959	-
	32171	-
	32213	-
N. sicca	Rush isolate	N. D.
N. polysaccharea	ATCC43768	N. D.
Eikenella corrodens	ATCC 23834	N. D.
Corynebacterium genitalium	ATCC 33030	-
Corynebacterium pseudotuberculosis	ATCC 19410	-
Corynebacterium xerosis	ATCC 373	-
Staphylococcus epidermidis	ATCC 12228	-
	ATCC 14990	-
	4233	-
	4234	-
	4235	-
	4236	-
Staphylococcus aureus	ATCC 33589	-
	ATCC 25923	-
	4241	-
	4247	-
	4248	-
	4249	-
S. auricularis	ATCC 33753	N. D.
S. saccarolyticus	ATCC 14953	N. D.
Streptococcus salivarius	ATCC 13419	-
	ATCC 7073	-
S. equi	NCTC 9682	N. D.
S. group G	4286	N. D.

STRAIN	REFERENCE	PROBE
		RDR140
		E. coli
Neisseria meningitidis	CMCC 2801	-
	ATCC 13077	-
Haemophilus influenzae	ATCC 33391	-
	2423	-
	503-1156	-
	503-1148	-
	503-1155	-
	503-1154	-
Streptococcus pneumoniae	ATCC 33400	-
	ATCC 6303	-
	4366	-
	4471	-
Escherichia coli	Strain B	+
	ATCC 11775	+
	9	+
	P3478	+
	2889	+
	340	+
Streptococcus agalactiae	ATCC 13813	-
	4352	-
	4353	-
	4354	-
	4355	-
	4356	-
Listeria monocytogenes	ATCC 15313	-
	G0282	-
	G0288	-
	F9784	-
	G0278	-
	F9841	-

Table 4

STRAIN	REFERENCE	PROBE
		RDR140
		E. coli
S. pyogenes	ATCC 19615	N. D.
S. dysgalactiae	ATCC 43078	N. D.
S. anginosus	ATCC 12395	N. D.
S. constellatus	ATCC 27823	N. D.
S. milleri	4224	N. D.
S. mitis	NCTC 3165	N. D.
S. mutans	ATCC 25175	N. D.
S. sanguis	ATCC 10556	N. D.
S. intermedius	ATCC 27335	N. D.
Bacillus subtilis	BD224	-
	6051	-
	558	-
B. cereus	11778	-
B. amyloliquefaciens	H	-
B. pumilis	ATCC 72	N. D.
B. brevis	ATCC 8186	N. D.
	ATCC 8246	N. D.
Propionibacterium acnes	ATCC 6919	-
P. avidum	ATCC 25577	N. D.
P. granulosum	ATCC 25564	N. D.
P. lymphophilum	ATCC 27520	N. D.
Flavobacterium meningosepticum	ATCC 13253	-

Claims

1. A probe capable of hybridizing to a bacterial nucleotide sequence containing a nucleotide sequence comprising at least 14 nucleotides and a sequence selected from the group consisting of

5'-GTACAAGGCCCGGGAACGTATTCACCG-3',
5'-GACGTCAAATCATCATGCCCCTTATGTC-3',
5'-GACGTAAGGGCCATGATGACTTGACGTC-3',
5'-GACGTAAGGGCCATGAGGACTTGACGTC-3',
5'-GGCGCTTACCACTTTGTGATTCATG-3',
5'-GACGTAAGGGCCGTGCTGATTTGACGTC-3',

a nucleotide sequence complementary thereto and mutants and fragments thereof.

2. A probe in accordance with claim 1, wherein the nucleotide sequence consists of about 20 to about 30 nucleotides.

3. A probe in accordance with claim 1 or 2, capable of hybridizing to a nucleotide sequence unique to bacteria and containing a nucleotide sequence selected from the group consisting of

5' GTACAAGGCCCGGGAACGTATTCACCG-3',

a nucleotide sequence complementary thereto and mutants and fragments thereof.

4. A probe in accordance with claim 1 or 2, capable of hybridising to a nucleotide sequence unique to gram-positive bacteria and containing a nucleotide sequence selected from the group consisting of

5'-GACGTCAAATCATCATGCCCCTTATGTC-3',

a nucleotide sequence complementary thereto and mutants and fragments thereof.

5. A probe in accordance with claim 1 or 2, capable of hybridizing to a nucleotide sequence unique to gram-negative bacteria and containing a nucleotide sequence selected from the group consisting of

5'-GACGTAAGGGCCATGATGACTTGACGTC-3',
5'-GACGTAAGGGCCATGAGGACTTGACGTC-3',

a nucleotide sequence complementary thereto and mutants and fragments thereof.

6. A probe in accordance with claim 1 or 2, capable of hybridizing to a nucleotide sequence unique to E. coli/enteric bacteria and containing a nucleotide sequence selected from the group consisting of

5'-GGCGCTTACCACTTTGTGATTCATG-3',

7. A probe in accordance with claim 1 or 2, capable of hybridizing to a nucleotide sequence unique to Bacteroides and containing a nucleotide sequence selected from the group consisting of

5'-GACGTAAGGGCCGTGCTGATTTGACGTC-3',

a nucleotide sequence complementary thereto and mutants and fragments thereof.

8. A primer capable of hybridizing to a nucleotide sequence unique to bacteria containing a nucleotide sequence with about 15 to about 30 nucleotides and comprising the sequence

5'-AGGAGGTGATCCAACCGCA-3' or
5'-AACTGGAGGAAGGTGGGGAT-3',

or a nucleotide sequence complementary thereto or a mutant or a fragment thereof.

9. A primer according to claim 8 containing a nucleotide sequence selected from the group consisting of

5'-AGGAGGTGATCCAACCGCA-3',
5'-AACTGGAGGAAGGTGGGGAT-3',

a nucleotide sequence complementary thereto and mutants and fragments thereof.

10. A method for determining the presence of a bacterial polynucleotide in a sample suspected of containing said bacterial polynucleotide, wherein said bacterial polynucleotide comprises a selected target region, said method comprising:
(a) amplifying the target region, if any, to a detectable level;
(b) incubating the amplified target region, if any, with a probe according to any one of claims 1 to 7 under conditions which allow specificity of hybrid duplexes; and
(c) detecting hybrids formed between the amplified target region, if any, and the probe.

11. The method of claim 10 wherein the target region is amplified by means of the Polymerase Chain Reaction (PCR).

12. The method of claim 10 or 11, wherein a pair of primers according to claims 8 or 9 are used to amplify the target region.

13. A PCR kit for the detection of bacteria comprised of a first container and a second container wherein the first container contains primers capable of amplifying a target region of a polynucleotide sequence within bacteria, and the second container contains one or more probes according to any one of claims 1 to 7.

14. A PCR kit in accordance with claim 13, wherein the first container contains a pair of primers according to claim 8 or 9.

FIGURE 1

Universal Bacteria Primers

Primer DG74

5'-AGGAGGTGATCCAACCGCA-3'

Primer RW01

5'-AACTGGAGGAAGGTGGGGAT-3'

FIGURE 2

Probe RW03 Specific for Gram-positive Bacteria

5'-GACGTCAAATCATCATGCCCCTTATGTC-3'

FIGURE 3

Probes Specific for Gram-negative Bacteria

Probe RW04

5'-GACGTCAAGTCATCATGGCCCTTACGTC-3'

Probe DL04

5'-GACGTAAGGGCCATGATGACTTGACGTC-3'

Probe DL05
5'-GTAAGGGCCATGATGACTTGAC-3'

Probe RDR278

5'-GACGTAAGGGCCATGAGGACTTGACGTC-3'

FIGURE 4

Universal bacterial probe - RDR 244

5'-CGGTGAATACGTTCCCGGGCCTTGTAC-3'

Universal bacterial probe - RDR245

5'-GTACAAGGCCCGGGAACGTATTCACCG-3'

FIGURE 5

Escherichia coli/enteric bacteria probe - RDR140KG

5'-GGCGCTTACCACTTTGTGATTCATG-3'

FIGURE 6

Bacteroides probe - RDR279

5'-GACGTAAGGGCCGTGCTGATTTGACGTC-3'

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 91 11 6396

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	WO-A-9 011 370 (HOLLAND BIOTECHNOLOGY) * claims * *	1-14	C 12 Q 1/68 C 07 H 21/00
A	JOURNAL OF BACTERIOLOGY vol. 164, no. 1, October 1985, BALTIMORE US pages 230 - 236; W. G. WEISBURG ET AL: 'Natural relationship between Bacteroides and Flavobacteria' * figure 1 * *	1-9	
A	FEBS LETTERS. vol. 94, no. 1, October 1978, AMSTERDAM NL pages 152 - 156; P. CARBON ET AL: 'The sequence of E. coli ribosomal 16 S RNA determined by new rapid gel methods' * figure 2 * *	1-9	
D,A	JOURNAL OF CLINICAL MICROBIOLOGY vol. 28, no. 9, September 1990, WASHINGTON US pages 1942 - 1946; H. W. KENNETH ET AL: 'Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction'		
A D	EP-A-0 272 009 (J. J. HOGAN ET AL) (& WO-A-8 803 957)		TECHNICAL FIELDS SEARCHED (Int. Cl.5)
P,A	US-A-4 977 251 (SALYERS ET AL) * the whole document * *	1,7	C 12 Q

The present search report has been drawn up for all claims

Place of search	Date of completion of search	Examiner
The Hague	10 December 91	MOLINA GALAN E.

CATEGORY OF CITED DOCUMENTS

X: particularly relevant if taken alone
Y: particularly relevant if combined with another document of the same catagory
A: technological background
O: non-written disclosure
P: intermediate document
T: theory or principle underlying the invention

E: earlier patent document, but published on, or after the filing date
D: document cited in the application
L: document cited for other reasons

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